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Virus protein transport across the gut epithelium of Spodoptera frugiperda (Lepidoptera: Noctuidae)

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ABSTRACT

A wide range of proteins move via vesicular transport across insect gut epithelial cells for release into the hemocoel. The utility of such transcytosed proteins for delivery of insect-specific neurotoxins from the gut into the hemocoel of aphids has recently been demonstrated for an aphid-transmitted, plant virus coat protein. Proteins that transcytose across the insect gut epithelium allow for appropriate delivery of toxins that are active within the hemocoel, providing a new approach toward development of pest resistant crops. We used an Ussing chamber to examine the transport efficiencies and mechanisms involved with the movement of a virus-derived coat protein and bovine serum albumin across the gut of *Spodoptera frugiperda*. Test proteins were labeled with fluorescein isothiocyanate or eGFP for ease of detection. There was wide variation in the efficacy of transport of the two proteins across the epithelial layer. The mechanisms involved with transport were investigated by addition of inhibitors into the Ussing chamber. While bovine serum albumen was taken up by clathrin-mediated endocytosis, uptake of the viral coat protein by the gut epithelial cell was dynamin dependent, but clathrin independent. Results are discussed in relation to the potential exploitation of these proteins and pathways for insect pest management.

CHAPTER 1: INTRODUCTION

Insect Resistance to Current Controls

The World in which we live is dynamic and undergoing rapid transformation. Recently, the United Nations FAO estimated that the world population will reach 9.7 billion people by 2050 and almost double by 2100 (1). A rapid increase in the demand for supplies will put pressure on an already over taxed system currently working to combat present deficiencies, such as poverty and malnutrition (1, 2). The agricultural industry, in particular, will face extraordinary demands for food and textile production, needing to ramp up production by some 50-70% (2, 4). Countries will have to navigate a decreasing rural labor force (2) and land availability, while providing more sustainable methods for agriculture. Global warming and the projected climate change will provide further stress on the agricultural community (3), notably increasing the spread of insects carrying crop and human diseases (4).

With about 10,000 species recognized as crop pests, insects and arthropods remain a concern for food security, as they continue to cause significant crop damage and promote the spread of plant pathogens (4). Insects annually destroy one fifth of the world's crop yields (5) and contribute to the billions of dollars lost to plant disease (4). In the United States, an estimated 13% of crops are lost to insect pests (4). Insects are responsible for the transmission of 76% of described plant viruses, with hemipteran insects transmitting a 55% majority (6). Plant pathogens account for $220 billion in economic losses (4). The severity of the impact on economic and agricultural sectors makes crop protection and pest management integral to future food security.

Current strategies to control herbivorous insects rely heavily on chemical pesticides and emerging transgenic technologies, such as plant expression of *Bacillus thuringiensis*-derived toxins. Pesticides can negatively impact non-target species, causing biological effects or fatality in non-target organisms [1, 2]. Despite development of resistance in over 500 species and environmental concerns, the United States spends $1.2 billion annually on pesticides [3], and this number is expected to inflate with the increasing range of pests due to climate shifts [4].

Insect-resistance transgenic crops to reduce impact of chemical insecticides

In the last few years research into improving crop protection strategies through the development of new insect resistance technologies relying on plant engineering has been extensive. However, agrochemicals have remained the most used method of insect control. Transgenic plants have been improved to be pest and herbicide-tolerant, and nutritionally rich [5]. Importantly, these molecular modifications to the plants are considered safe for mammalian consumption, and contribute to the reduction of insecticide use.

Transgenic Bt crops were first introduced in 1996, and the numbers of acres planted has increased annually. By 2003, the United States had adopted transgenic soybean, corn, cotton, canola, squash and papaya and saw increases in production yields and income [6]. The use of transgenic technologies has also reduced the use of pesticides, in some areas up to 70% saving billions of dollars associated with chemical purchase and application [6]. Since initial adoption, insect-resistant transgenic corn usage has grown to 79%, cotton to 84%, and genetically engineered soybean to 94% within the US [7]. Worldwide, transgenic crops spanned over 181.5 million hectares in 2014 and were adopted by 28 countries [5]. However, due to lack of

conformance with refuge planting and coincident use of transgenic cultivars, field-evolved resistance has been reported in both Bt cotton and corn [8, 9].

Despite this, engineered crops with specific insecticidal modes of action have shown greater ability to defend against feeding damage caused by pests, than sprayed chemicals. However, reliance on a single successful control method will eventually render it useless. Integrated pest management (IPM) is needed to reduce the risk, or delay the development of insect resistance. Multiple approaches to pest control are required for IPM, such as expression of multiple toxins with different modes of action, investigation into novel target sites, and an influx of new active compounds. One such avenue is the discovery and use of new active bioinsecticides that are abundant in the environment.

New Methods to Pest Control: Bioinsecticides

There are a number of peptides, hormones and neurotoxins that are insect-specific and act within the insect hemocoel (body cavity) but lack appropriate delivery systems for use in pest control. Organisms, including microbes (bacteria and fungi), arthropod predators (spiders, wasps and parasitoids), insects and entomopathogenic nematodes provide a wealth of untapped insect-specific toxins and peptides that have unique targets with a wide range of selectively insecticidal activities. As many of these toxins act within the insect hemocoel, their application in the field is complicated as they cannot readily penetrate insect barriers (i.e. cuticle and gut). Two important sources of insect specific bioinsecticides that have shown potential as crop protectants are arachnid venom and insect-derived neuropeptides.

Spider toxins

Arachnids have highly complex venoms with numerous toxic peptides to paralyze and kill prey quickly; many of these toxins have insecticidal activity. It is estimated that about 10 million peptides are present in arachnid venoms, with 0.5-1.5 million being selectively insecticidal [10-12]. Many of these arachnid venoms are rich in ion channel modifying neurotoxins that cause rapid mortality on contact with neurons. The cysteine-knot is a conserved structural motif found in many spider toxins, with two disulfide bonds forming a ring and a third disulfide penetrating the ring in a pseudo-knot [11, 13]. Toxins such as the omega atracotoxin Hv1a from the Australian funnel-web spider have cysteine-knots. This structure provides a high level of stability and resistance to proteases making these toxins attractive for stable transgenic expression or for soil application.

Spider venom-derived peptides provide small stable peptides with no known toxicity towards vertebrates, and target a wide range of arthropod pests. However, they are largely inefficient when delivered orally. Some commercial cysteine-knot peptides are sold as spray applications (Vestaron Corporation), but rely on Bt toxin-mediated disruption of the midgut for access to the hemocoel where they exert insecticidal properties. To reduce the reliance of future products on Bt, spider and other insect-specific toxins from snakes, wasps and bees will need appropriate delivery to their target sites within the hemocoel of the targeted pest. Appropriate delivery is critical for their adoption for crop protection.

Insect-derived hormones and peptides

Bioinsecticidal toxins can also be derived from insect sources – hormones, neuropeptides, and enzymes – which are synthesized *in vivo* and can be used to target very

specific activities unique to arthropods. There are a number of neuropeptide and hormone families discovered across multiple insect orders that are vital in regulating physiological processes, briefly: adipokinetic hormones (metabolic functions, locomotion, cardiostimulation), diuretic hormones (osmotic and feeding behaviors), prokinins and myokinins (muscle stimulation), and allatostatins (inhibit juvenile hormone synthesis) [14-17]. As these neuropeptides and hormones are present in many insect species and have antagonistic effects on biological functions when inappropriately expressed, they have received attention as new agents for insect control. These insect-derived peptides function within the hemocoel and are effective on injection, inducing mortality at low doses [18]. Peptide hormones and neurotoxins lack oral and topical effectiveness due to their inability to penetrate the cuticle or gut wall and rapid degradation by peptidases [19, 20].

Potential delivery agents for toxins that act within the hemocoel

The adoption of spider- and insect-derived toxins that act within the insect hemocoel to control pest populations relies on the development of novel delivery systems that are both effective and insect-specific. There is a wide range of proteins that move across the gut barrier via active vesicular transport (transcytosis) for release into the hemocoel [21-23], including serum albumin, plant-derived lectins and viruses. Such proteins could potentially serve as delivery vehicles to deliver peptides and toxins from the gut lumen to their target site within the hemocoel. Bovine serum albumin was selected for analysis of the efficiency of movement across the insect gut for comparison with virus-derived proteins.

Albumin

Albumin, an abundant serum protein, functions in a vast number of processes in humans. Albumin binds endogenous and exogenous proteins, transporting many of them across mammalian epithelia [24]. In insects, bovine serum albumin (BSA) has also been found to mediate the movement of proteins across the gut [25, 26]. In the aphid, BSA can increase the movement of a plant polerovirus into the hemocoel [27], possibly through the binding and subsequent exploitation of the BSA receptor. The extensive study of albumin has revealed its propensity to use multiple endocytic mechanisms and receptors across a wide range of mammalian tissues to traverse barriers [24, 28-33]. The ability of albumin to facilitate protein movement across the insect gut provides a useful comparison for more insect-specific proteins and their transport.

Ussing chamber to assess protein transport

Epithelial monolayers can use two routes to transport cargo: paracellular, transport between cells and transcellular, transport within the cell to the opposite surface. Transcytosis is an active form of transcellular transport and involves endocytic mechanisms (fluid-phase ingestion, non-specific uptake, and receptor-mediated uptake) to internalize macromolecules, particles and fluid into vesicles. These vesicles traffic cargo across polarized cellular barriers, bypassing lysosomes and releasing contents at the opposite surface. Fluid-phase transcytosis is characterized by smooth vesicles and the bulk uptake of water-soluble molecules [28]. Cells can selectively recognize and transport certain molecules via receptor-mediated transcytosis, with vesicle properties dependent on the cargo, receptor(s) and tissue. Transcytosis in mammals is

well characterized, but there has been limited research into the underlying mechanisms of transcytosis in insect systems [21, 26, 34, 35].

One method to study the movement of ions, drugs, and proteins that transport across epithelial tissues is with an Ussing Chamber. Developed by Hans Ussing in the 1950s to study the active NaCl transport in frog skin [36, 37], the chamber is an *ex vivo* physiologically relevant system where dissected tissue separates two chambers of identical solutions. In this set up the pinned tissue acts as the gateway between chambers, and when a drug, nutrient or protein of interest is added to one chamber its movement can be measured to the other. Identical solutions and volumes help eliminate passive movement through paracellular pathways [38]. The Ussing chamber has aided in drug transport studies as it provides a system that better mimics *in vivo* conditions, than cell culture [39, 40].

The Ussing chamber has been used primarily with mammalian epithelia, with a large focus on gastrointestinal physiology and transport. Researchers have used the chamber to investigate intestinal barrier function, which plays a role in pathogen defense both in mammals [41, 42] and in insects [43]. The Ussing chamber has also aided investigation into the effects and uses of viruses to alter epithelial function for treatment of human diseases [44-47]. Since its introduction, the Ussing chamber has provided a proven method to study physiological transport processes, but adaption of the system to insects has been slow. In the 1970s, Wood and colleagues studied ion transport across lepidopteran larval midguts, performing the first insect chamber experiments [48-51]. Later Casartelli et al [26] studied the movement of large proteins across the midgut of the silkworm, *Bombyx mori*, and Multeau et al [52] and Wang et al [53] investigated the movement of viruses that naturally overcome the midgut barrier to

establish infection. With increasing interest from the agricultural sector in the delivery of active toxins to the insect body cavity, the Ussing chamber provides a controlled environment from which to study proteins that traverse the cellular environment.

Viruses for toxin delivery

Viruses have evolved to successfully penetrate cellular barriers to establish infection or further transmission. There is a long list of viruses that either primarily replicate in insects, or use insects as vectors to reach new hosts. Viruses that cross the gut epithelium to reach the hemocoel that are insect restricted could be exploited to deliver toxins to their target sites within the hemocoel. The coat proteins that enable specific receptor-mediated entry and trafficking could create a new line of biological control agents that are safe for vertebrate consumption.

Work with Pea enation mosaic virus (PEMV) and Tobacco mosaic virus (TMV) has proven the potential benefit of using viruses to control insects. PEMV is a plant virus with a single stranded, positive sense RNA genome that uses receptor-mediated transcytosis to enter the aphid body cavity [54-56]. PEMV then traffics to the salivary glands for release and spread to new plant hosts during aphid feeding [54]. This naturally evolved mechanism of entry into the hemocoel was exploited when the luetovirus coat protein was fused to Hv1a (spider toxin) and fed to aphids, causing mortality [57]. In another case, the trypsin modulating oostatic factor (TMOF) produced natural in mosquitos, shows limited toxicity when fed to lepidopteran larvae. Fusion to the TMV coat protein created virus particles expressing TMOF on the surface, and drastically reduced the amount of TMOF needed to induce mortality [58]. This demonstration of oral delivery facilitated by a virus coat protein could be exploited in other insect systems.

It is well known that many plant viruses hijack intracellular pathways to cross the gut and salivary gland. The insect densoviruses, Junonia coenia densovirus and Galleria mellonella densovirus can cross the midgut without replicating, and cause infection in subepithelial layers. Junonia coenia densovirus in particular, is fatal to a number of lepidopteran species. It is hypothesized that the structural proteins that modulate midgut trafficking and release of this virus into the hemocoel could also carry insecticidal peptides. Our primary goal is to investigate the mechanisms for JcDNV structural protein movement across the gut epithelium of a lepidopteran host, toward potential used of these proteins for toxin delivery.

Densovirus: Insect Pathogens

The expression “Virose à noyaux denses” (viral disease resulting in dense nuclei) was first used in 1966 by the Laboratoire de Pathologie Comparée to describe a symptom plaguing the greater waxmoth, *Galleria mellonella* [59]. These viruses were later named densovirus due to the nuclear hypertrophy exhibited during infection called densonucleosis. Densoviruses are classified in the subfamily *Densovirinae* within the family *Parvoviridae*. They are small non-enveloped single-stranded DNA viruses with a diameter of 18-22 nm [60, 61]. The five genera in the subfamily *Densovirinae* (*Ambidensovirus, Brevidensovirus, Hepandensovirus* and *Penstyldensovirus*) have either ambisense or monosense genomes with 4-5 structural proteins and replicate in almost all insect tissues.

After initial discovery in *G. mellonella* [62], over 30 densoviruses have been found that are highly pathogenic to species within a number of insect orders including Hemiptera, Lepidoptera, Dictyoptera, Odonata, Diptera, Orthoptera [63, 64] and in some cases Decapoda [65-67]. More recently, densoviruses have been identified in aquatic systems with potential

members discovered in sea urchins (Echinodermata) [68] and freshwater crayfish (Decapoda) [69]. A densovirus, aptly named sea star-associated densovirus, has been implicated as the pathogen responsible for the extensive outbreak of sea-star wasting disease along the North American Pacific coast [70].

Until this year, members of the subfamily *Densovirinae* have been known only to infect invertebrates, while the subfamily *Parvoviridae* target mammals and birds. However, the genome of a densovirus tentatively named human CSF-associated densovirus 1 (HuCSFDV1) has been found in cerebrospinal fluid of a human with encephalitis [71]. Densoviruses have not been shown to infect vertebrates, and the research group cautions that HuCSFDV1 has not yet been confirmed to replicate in humans and briefly speculate on possible sources of contamination [71]. Until replication of the virus in a human host is definitive, the expansion of *Densovirinae* host range is limited to invertebrates [72].

Junonia coenia densovirus

Junonia coenia densovirus (JcDNV) (genus *Ambidensovirus*) was first isolated from the *Junonia coenia* (Common Buckeye) moth. JcDNV has a linear 6 kb ssDNA ambisense genome flanked by identical long (>500 nt) inverted terminal repeats packaged into icosahedral capsids [73, 74]. JcDNV has terminal Y-shaped hairpin structures that serve as primers and are essential for the rolling-hairpin mechanism used in replication [73]. During virion production equal amounts of positive and negative strand DNA are encapsidated [75] and both strands contain ORFs for the nonstructural (NS) and structural proteins. One strand has ORF2, ORF3, and ORF4 with an upstream P93 promotor that encodes the nonstructural proteins NS-1, NS-2 and NS-3, respectively (Figure 1). NS-3 is essential for JcDNV replication in the permissive cell line, LD652

and in *Spodoptera littoralis* [76], while NS-1 is the typical helicase superfamily III [77]. The complimentary DNA strand encodes all four overlapping structural proteins VP1, VP2, VP3 and VP4 in-frame in one long ORF under the control of a P9 promotor [78]. Translation of ORF1 produces VP1 and VP2-4 are synthesized via leaky scanning along the mRNA [74]. Thus, one ORF produces 4 coat proteins that vary in size from 40-100 kDa with the same C-terminal region [79]. Each coat protein begins translation at an AUG codon (two AUG codons positioned 6 nucleotides apart initiate VP4 translation), and capsids are constructed from 60 copies of the polypeptides for an approximate ratio of 1:9:9:41 (VP1:VP2:VP3:VP4) [74, 80].

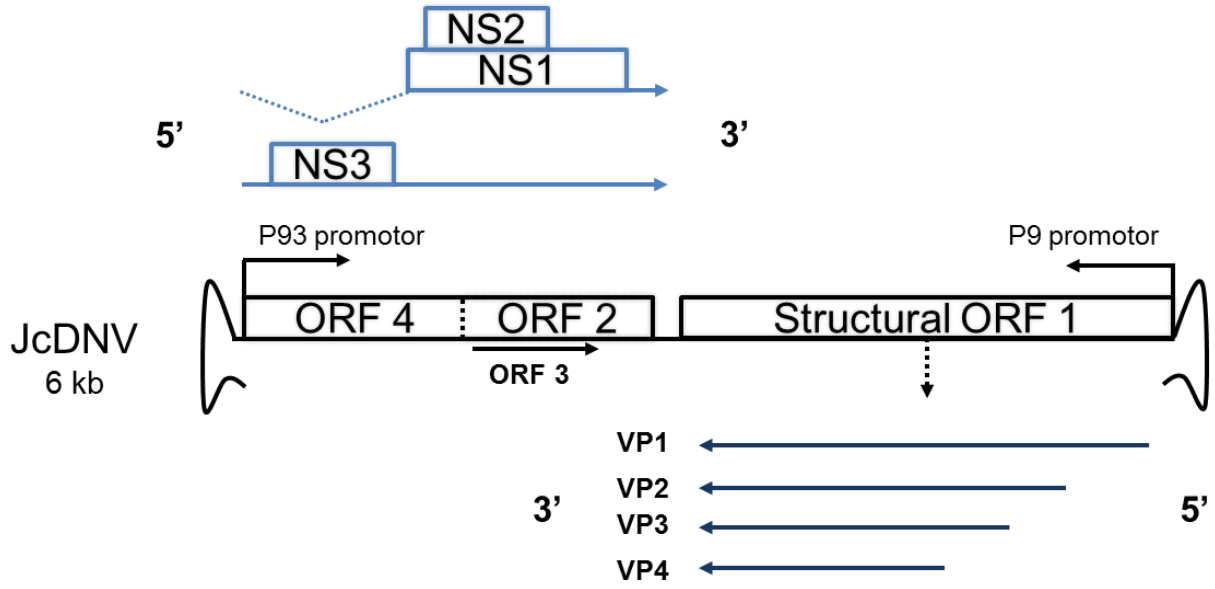

Figure 1. Genome of Junonia coenia densovirus depicting the open reading frames (ORF) of non-structural (NS) and structural proteins (VP), produced by leaky scanning and alternative splicing.

JcDNV host range

The extent of the host range varys greatly among densoviruses. The first densovirus characterized, *Galleria mellonella* densovirus (GmDNV) is monospecific, with pathogenicity only

to the greater waxmoth. JcDNV shares a high level of sequence homology to GmDNV [61], but has a much broader host range and is reported to be pathogenic to *Spodoptera littoralis, Spdoptera frugiperda, Mamestra brassicae, Bombyx mori, Junonia coenia,* and *Lymantria dispar* [81-83]. Notably, JcDNV can not establish infection in *G. mellonella* [81].

Densovirus entry and infection

The tissue tropisim of densovirues also varies with almost all tissues found to support virus replication. Both GmDNV and JcDNV infect multiple insect tissues, but notably avoid replication in the midgut [82, 83]. The signal(s) or mechanism(s) by which JcDNV avoids replication while crossing the midgut barrier are currently unknown. After traversing the intracellular environment, JcDNV quickly establishes infection in the trachea overlying the gut and in hemocytes in *S. frugipera,* eventually leading to infection of the epidermas and host death [82]. Mutuel et al (2010), estimated that only 0.1% of JcDNV is able to complete this journey, citing the midgut as an almost intractable barrier to infection. However, *ex vivo* infection experiments using *S. frugiperda* gut epithelium showed efficient virus movement into the hemocoel [53]. The peritrophic membrane is not expected to limit virus exposure to the midgut due to the relatively small size of the virus. Binding to appropriate receptors on the gut epithelial surface is likely to be a primary determinant of host range.

Many parvoviruses have been shown to enter cells via receptor-mediated endocytosis [84-86]. The viral capsid mediates host specificity through binding to one or more receptor molecules expressed on specific cell surfaces [87]. Parvoviruses that infect vertebrates tend to share common capsid features, most notably a depression around the fivefold axis, a dimple on the twofold axis and spike(s) on the threefold axes. Evidence has suggested that the threefold

axis spikes are sites of receptor binding [88, 89]. The parvovirus, feline panleukopenia virus (FPV) is infamous for its leap in the 1970s from cats to dogs becoming known as canine parvovirus (CPV). Only a few years later CPV underwent a further host change and became highly virulent to both cats and dogs. The ability to recognize and bind the dog transferrin receptor was responsible for this rapid host shift, and was due to three mutations of the threefold axis spike on the viral capsid surface [90].

In contrast to the vertebrate family of parvoviruses, JcDNV has a relatively smooth capsid with two small spikes. GmDNV and JcDNV share 96% similarity in VP4, the most abundant protein in the capsids. Using VP2 and VP4 virus-like particles (VLP) [79], 8 amino acids were found to be different between the densoviruses, resulting in conformational changes to the larger capsid spike and the fivefold axis cylinder on JcDNV [74]. Substitution of these eight amino acids for GmDNV residues showed that the fivefold axis determined midgut tropism [52]. Mutant JcDNV did not show reduced virulence when injected into the hemocoel, suggesting that host specificity is determined by capsid recognition of midgut receptors.

In the permissive LD652 cell line derived from the gypsy moth, *Lymantria dispar*, JcDNV and VP4 VLPs are rapidly endocytosed via clathrin coated vesicles, with slower trafficking to the nuclei where they must wait for S phase to replicate [91]. Virus particles were found in clathrin-coated pits using electron microscopy, and clathrin inibitors prevented cellular entry. However, *ex vivo* transport of JcDNV across *S. frugiperda* midguts was found to be reliant on dynamin-dependent mechanisms, and not clathrin-mediated endocytosis [53]. The exploitation by the virus of multiple mechanims or of CLIC/GEEC (clathrin-independent carriers / glycosylphosphatidylinsositol) and bulk-phase endocytosis can not be ruled out. Use of multiple

lepidopteran species challenged with the Gm residue-mutant and wild-type JcDNV suggests a conserved mechanism of transcytosis [53].

JcDNV and other parvovirues depend on undefined elements of the cytokeleton network for entry and trafficking [91, 92]. After entry, escape from the endosome may involve the conserved Phospolipase A_2 (PLA_2) activity on VP1, however removal of PLA_2 does not abolish infection. Parvovirues are highly virulent to suceptible organisms but infection tends to be inefficient. Indeed, this could be the case with JcDNV where the rate limiting step may be escape from the endosome [82, 91, 93, 94].

JcDNV structural proteins

The majority of parvoviruses have a conserved region of 39 amino acids in the N-terminal extension of VP1, found to be important for infection [95-97]. Densoviruses that have very little sequence homology among structural proteins, can have around 90% identity within this domain [64, 98]. This region exhibits PLA_2 activity belonging to a new group within the superfamily, known as group XIII [95]. While the parvovirus PLA_2 differs from secretory PLA_2 in sequence, structure and biological role, the catalytic site (HDXXY motif) and Ca^{2+} binding loop (YXGXG motif) remain conserved. Zádori et al (2002) determined that VP1 was not involved in virus entry, but a conformational shift leading to its externalization allowed the virus to escape endosomes and establish infection.

Expression of the structural ORF1 produces JcDNV capsids that are composed of 60 copies of polypeptides. Structural protein 4 (47 kDa) is the major capsid protein and is capable of forming VLPs morphologically similar to the wild type virus [99]. VP2 and VP3 can also form pseudocapids when expressed with VP4, indicating VP4's role in coat assembly. Importantly,

VP4 VLPs are capable of mimicing entry and transport in cell culture and the NS proteins are not required for capsid formation [91]. VP1 is unstable when expressed on its own due to sequence rearrangement [99].

Densoviruses for biological control

Densoviruses have been considered as pest control agents since their intial discovery. GmDNV infected *G. mellonella* larvae were used to eradicate these pests from beehives [100]. Densoviruses have qualities attractive to pest management, such as high virulence and limited host range. These viruses are also stable after isolation in the laboratory and upon long term storage. They can persist naturally and continue to establish infections in insects through vertical and horzontal transmission. Concerns over sequence similarity to the common ancester of vertebrate parvoviruses have been raised . However, invetebrate densoviruses have yet to cause infection in a vertebrate host [101].

The ability of VP4 to form morphologically correct particles without the need of additional proteins, could provide for an attractive delivery system for pest management. The use of double stranded RNA, for RNA interference (RNAi) in insects has great potential as a crop protection strategy [102-108], however delivery of RNA into some insects is inefficient [109]. Viruses are particularly attractive as delivery agents, for their natural ability to gain entry across cellular barriers, indeed virus vectors for the delivery of siRNA have been investigated [110, 111].

The convenient ability of VP4 to self-assemble and potential to traverse barriers makes it attractive for use in pest management, either as a fully formed capsid or as a fusion protein.

Host limitation and high virulence are favorable for use of VP4 as carriers of a wide array of control agents.

Other densovirus applications

Densoviruses with high specificty to mosquito species have been found in many mosquito cell lines [101, 112, 113], inspiring their use as alternative vector control agents to counteract the spread of human diseases [114-116]. Liu et al (2016) were able to produce a recombinant Aedes aegypti densovirus that stably induced miRNA-based gene silencing *in vivo*, which has potential application to contol the *Aedes aegypti* vector of Dengue virus. Work with insect cell lines and JcDNV, has shown the ability of JcDNV-based vectors to integrate genes into lepidopteran genomes for expression, providing a new tool for the production of transgenic cells or insects [117, 118].

Exploration into Novel Delivery Strategies

There is a need to increase our understanding of the physiological transport processes in the insect gut, while identifying viable methods that can exploit native cellular functions for novel insect control practices. To this end the hypotheses to be addressed in this thesis are:

1. JcDNV uses receptor-mediated endocytosis to traverse the midgut of its host, *Spodoptera frugiperda.*
2. The transport efficiency of the structural protein VP4 across the gut epithelium is efficient.
3. The major capsid protein, VP4, can be used to deliver toxins that act within the hemocoel to target lepidopteran pests of agriculture.

Thesis Organization

In Chapter 2, we report on our use of an Ussing Chamber to study the *ex vivo* movement of albumin and JcDNV VP4 (fused to eGFP) across the midgut of the agricultural pest, *Spodoptera frugiperda*. In chapter 3 we discuss more in-depth the significant findings and their implications for future research directions, in particular, the potential use of VP4 for toxin delivery.

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CHAPTER 2: PROTEIN TRANSPORT ACROSS THE GUT EPITHELIUM OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE)

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Abstract

The increased incidence of insect resistance to current control methods has spurred investigation into alternative approaches to pest control with emphasis on health and environmental safety. Insect-specific toxins and peptides that act within the hemocoel of the insect and are not active on ingestion provide one such avenue. Proteins that transcytose across the insect gut epithelium could provide effective delivery for such hemocoel-active peptides providing a new approach toward development of pest resistant crops. In this study, we used an Ussing chamber and endocytic inhibitors to characterize the transport of a structural protein of Junonia coenia densovirus and of bovine serum albumin across the midgut of the economically important pest, the fall armyworm (*Spodoptera frugiperda*). Albumin, which has been well characterized, provided a reference protein for the study of the viral structural protein. Albumin flux was found to be clathrin-mediated with a transport of 525 pmol/cm^2 after 2 hours with evidence for intracellular degradation. Albumin was detected in epithelial columnar cells by immunofluorescence microscopy. The densovirus coat protein efficiently crossed the midgut with a rate of 60 pmol/cm^2/2h and was dependent on membrane fusion. Ligand blots were used with each test protein to assess the specificity of binding to gut epithelial proteins.

Introduction

The fall armyworm (*Spodoptera frugiperda*) (J.E. Smith), is a polyphagous pest of agriculture throughout the western hemisphere, with a large native range from Argentina to central Canada. *S. frugiperda* are voracious feeders consuming a vast range of about 100 plant species [1], including major economic crops. Agrochemicals effective against *S. frugiperda* are limited and require multiple costly applications [2]. Although transgenic plants expressing Bt toxins showed initial promise at providing protection against *S. frugiperda* [3-6], resistance has been reported since the early 2000s [4-7], and more recently to Cry1F in TC1507 corn [3, 8].

Exploitation of biological toxins and peptides with highly selective insecticidal activity may counteract the drawbacks associated with use of conventional pesticides [9, 10]. For example, arachnid venoms are the source of some 1.5 million highly stable, insecticidal peptides that remain untapped for pest control. In addition the use of insect-derived hormones and neuropeptides for pest management is appealing as they target key functions in growth and development [11, 12]. Importantly, these neurotoxins and peptides provide a breadth of unique target sites and actions that could be used to control chemically-resistant pests.

However, these agents require effective delivery to their target sites located within the body cavity. Proteins found to transcytose (i.e. vesicle-mediated transcellular transport) across the polarized gut epithelium of lepidopteran and hemipteran insects have been investigated for potential use as toxin delivery agents [13, 14]. Information on protein transcytosis in the insect is limited. Proteins that bind specifically to the insect gut and that transcytose across the insect gut epithelium may provide for new vehicles for delivery of such toxins.

Some viruses have evolved to transcytose across the gut barrier and establish infection, and/or to circulate to the salivary glands for subsequent transmission [14-16]. The genus *Ambidensovirus* (*Parvoviridae)* encompasses viruses with small, single-stranded DNA genomes that are constrained to insect hosts and includes *Junonia coenia* densovirus (JcDNV) [17, 18]. JcDNV rapidly crosses the gut monolayer after oral ingestion without undergoing replication, ultimately causing mortality in *S. frugiperda* [19, 20]. Virus proteins, such as the structural protein of JcDNV that crosses the gut epithelium have potential use as novel delivery agents for pest control [19, 21-23]. JcDNV is hypothesized to use receptor-mediated transcytosis, similar to other parvoviruses [24-27], and it is expected that the major capsid protein (VP4) expressed individually will behave in a similar fashion.

Albumin, a serum protein, has been well characterized. Albumin crosses mammalian endothelial barriers by receptor-mediated transcytosis [28-35], and has become medically important in promoting stable drug delivery [30]. Albumin also uses receptor-mediated clathrin-dependent endocytosis in *Bomby mori* [36, 37] and facilitates the endocytosis of a plant virus by an unknown mechanism [38]. Albumin was used in the current study of protein transcytosis as a reference protein for comparative purposes.

We analyzed the ex vivo movement of the JcDNV structural protein, VP4 with bovine serum albumin (BSA) as a reference protein, across the gut epithelium of *Spodoptera frugiperda* within an Ussing chamber. Our investigation into the transport efficiency and endocytic mechanisms involved increases our understanding of transcytosis across the lepidopteran gut and allows for assessment of the potential utility of VP4 as a toxin delivery vehicle for use in agricultural pest control.

Materials and methods

Insect rearing

Spodoptera frugiperda (corn strain) eggs were obtained from Dr. Robert L. Meagher, USDA-ARS CMAVE, FL, and larvae were reared in a growth chamber (25°C, 16h light, 8h dark period) on a wheat germ- and soy flour- based artificial diet (Frontier Scientific, General Purpose Lepidopteran diet).

Proteins and JcDNV Viral Protein 4 construct

To test macromolecular transport across the epithelium of *S. frugiperda,* bovine serum albumin (BSA; albumin) labelled with fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich. The bacterial expression vector pBad-HisB containing eGFP was transformed into z-competent Top10 *E. coli,* and expression was induced with 0.02% L-(+)-Arabinose (Sigma-Aldrich) overnight at ambient temperature. Ni-NTA agarose resin (Qiagen) was used to purify eGFP following manufacturers' protocol.

The cDNA sequence encoding Junonia coenia densovirus (JcDNV) structural protein VP1 (GenBank accession number: NC_004284.1) fused to a proline rich linker and eGFP (JcDNV VP1-P-eGFP) was synthesized by GenScript. PCR was used to amplify the VP4 region within VP1 (Fig 1). The forward primer was modified from Croizier et al [39]. To obtain the VP4 sequence, primers VP4 F (5'- ATCGTAGGATCCGCT*ATG*TCATTACCTGGAACTGG-3') and VP4 R (5'-ACTGAGAAGCTTTAG*TTA*GCCGCTTTACTTGTACAG-3') were used. The PCR fragment was digested with BamHI and HindIII (Promega) and cloned into pGex-2T. A C terminal 6xHis tag was added

using PCR and the primers VP4 F, VP4 R1 (5'-ATCTTAAAGCTTTTAGTGATGGTGATGGTGATGACCAGAGCCGCCGCTTTACTTGTACAGCTCGTCCAT-3') and VP4 R2 (5'- ATCTTAAAGCTTTTAGTGATGGTGATGGTGATGACCAGAGCC -3'), and this fragment was digested and cloned into Bac-to-Bac® Baculovirus Expression System (Invitrogen) donor plasmid pFastBac1.

Baculovirus expression of VP4-P-eGFP and purification

Cells (SF9) maintained in Sf-900™ III serum free media (Gibco) without antibiotics were transformed with the recombinant bacmid using Cellfectin (Invitrogen) to produce recombinant VP4-P-eGFP. Expression of VP4-P-eGFP in insect cells was confirmed using fluorescence microscopy to detect eGFP fluorescence (excitation wavelength 395 nm, emission wavelength 488 nm) and standard western immunoblotting procedures using rabbit anti-eGFP (1:5000) and goat anti-rabbit (1:5000) (Supplementary Fig 1). For protein expression, 200 ml of SF9 suspension cell culture was infected with recombinant baculovirus VP4-P-eGFP for 1 hour, after which fresh medium was added to a final volume of 400 ml (1-2x10^6 cells/ml). Suspension cultures were incubated at 28°C, shaking at 140 rpm. As VP4-P-eGFP clustered within the cells, indicative of insolubility, the fusion protein was extracted from the cells at 72 hours post infection and denatured as described in [40]. The final solubilization step using 6M Guanidine-HCl was not performed, as the majority of VP4-P-eGFP was solubilized using 8M Urea. Protein was refolded by slow dialysis, using a decreasing concentration of Urea (4, 2, 1, 0.5, 0 M) in each successive buffer containing 50 mM Tris and 300 mM NaCl, pH 8.0. Each buffer exchange was performed at 4°C 24 hours, and the final buffer without Urea was repeated twice.

Refolding was assessed by observation of the fluorescence of eGFP and lack of protein aggregation after centrifugation at 4000 rpm 15 minutes. Refolded VP4-P-eGFP was concentrated using PEG20 and Slide-A-Lyzer™ dialysis cassettes (ThermoFisher Scientific). A Bradford assay was used to quantify proteins. Denaturation and refolding protocols removed about 80% of extraneous Sf9 proteins.

Preparation of lepidopteran brush border membrane vesicles

Brush border membrane vesicles (BBMV) of 14-20 sixth instar *S. frugiperda* were prepared from isolated midguts as described previously [41]. Final aliquots of BBMVs in cold diluted 1:2 MET buffer (0.3M Mannitol, 5 mM EGTA, 17 mM Tris-HCL pH 7.5) were stored at -80°C and used within two months of isolation. Protein concentration was determined by Bradford assay [41]. An average 7-13-fold enrichment in aminopeptidase activity of the final BBMV preparations from the initial homogenate was measured as described in [41].

Two-dimensional gel electrophoresis and Ligand Blotting

Suspensions containing 50 µg of midgut BBMV proteins were separated using two-dimensional gel electrophoresis as described in [41]. After centrifugation to remove insoluble material, the proteins were applied to a 7 cm Immobiline DryStrip gel, pH 3-10 (GE Healthcare) for a two hour rehydration. An Ettan IPGphor 3 (GE Healthcare) was used for isoelectric focusing as follows: 50V (10 h), 500V (1 h), 1000V (1 h), and 8000V (10 h). Following isoelectric focusing, BBMV proteins were separated by size using mini-protean TGX precast gradient gels (BioRad), run at 180V in cold buffer for 50 minutes.

After two-dimensional separation, gels were either silver stained according to the manufacturer's protocol (BioRad Silver Stain) or transferred to a nitrocellulose membrane for one hour at 100V. Transferred membranes were incubated for one hour at room temperature with 5% non-fat dry milk in phosphate buffered saline with 0.2% Tween-20 (PBST). If blots were to be exposed to albumin, 0.5% polyvinyl alcohol was substituted for milk. Blots without ligand probing were used to determine if antibodies targeted unbound BBMV proteins, and eGFP was used as a control for VP4-P-eGFP. Blots were probed with 5 nM of Albumin or eGFP in PBST for one hour at room temperature. Primary antibodies (anti-BSA (1:5000), anti-GFP (1:5000)) were used to detect bound ligands followed by HRP-conjugated secondary antibodies (Goat anti-rabbit (1:10000)). This allowed for detection using HyGLO chemiluminescent HRP detection reagents and X-ray film following standard procedures. Experiments were repeated twice for each ligand.

Midgut isolation

The midguts of sixth instars were isolated for perfusion studies. Briefly, larvae were sedated on ice for 30 minutes prior to dissection. The midguts at this instar are large enough to mount on sliders without tissue perforation. The dissection was performed in cold Insect Physiological Solution (47 mM KCl, 20.5 mM $MgCl_2$, 20 mM $MgSO_4$, 1 mM $CaCl_2$, 88 mM Sucrose, 4.3 mM K_2HPO_4, 1.1 mM KH_2PO_4, adjusted to pH 7.5) [42]. The larval gut was exposed by opening the cuticle via a longitudinal incision on the ventro-lateral side. The midgut was isolated excluding the anterior and posterior regions, opened longitudinally and mounted on

sliders (0.1 cm^2) for transport studies using an Ussing chamber (Physiologic Instruments, Model P2300). Peritrophic membranes were removed for these experiments.

Ussing Chamber experiments

The midgut epithelium mounted in the Ussing chamber was perfused with 2.5-3 ml of luminal buffer (5mM CaCl2, 24 mM MgSO4, 20 mM potassium gluconate, 190 mM sucrose, 5 mM CAPS, pH 10.0) in the lumen compartment and 2.5-3 ml of hemolymph buffer (5mM CaCl2, 24 mM MgSO4, 20 mM potassium gluconate, 190 mM sucrose, 5 mM Tris, pH 7.0) in the hemocoel compartment. A continuous supply of oxygen was provided to both chambers, and experiments were performed at 27°C protected from light. FITC-Albumin (15 uM), eGFP (3 uM) and VP4-P-eGFP (3 uM) were added to the lumen chamber. FITC-albumin was run through a gel matrix that removed unbound FITC, immediately before experimentation. Samples of 100 µl were collected at 10, 20, 30, 60, and 120 minutes. Total chamber volumes were collected after the 120 minute time point, and analyzed via standard western immunoblotting procedures to determine integrity of proteins in the lumen and hemocoel chamber.

Time course samples were analyzed using a fluorescence microplate reader (excitation wavelength 495 nm, emission wavelength 525 nm). Standard curves with known concentrations of the test protein in hemolymph buffer were used with each 96-well plate to calculate the amount of protein transported across the epithelium. The protein flux was expressed as pmol/cm^2, taking into account solution volume, molecular weight and tissue area (cm^2). Rates of transport were calculated using the time course samples and expressed as pmol/cm^2/2h. Flux experiments were repeated with a minimum of 6 midguts per protein, and inhibitor

experiments with a minimum of 4 midguts per treatment. Mean ± standard error is provided for flux and rates of each protein.

Experiments were run for no longer than 2 hours to ensure that tissue remained viable in the chamber during the course of each experiment. Sections of paraffin-embedded tissue taken from chamber experiments displayed healthy epithelium morphology with no apparent loss of integrity.

Addition of endocytic inhibitors

Endocytic inhibitors (chlorpromazine (CPZ), methyl-β-cyclodextrin (MBCD), dynasore (DYN) and tannic acid (TA)) were added to the luminal compartment 30 minutes prior to the addition of protein and were maintained in the chamber throughout the experiments. Dynasore was not used in experiments with albumin, as this drug has been shown to bind serum proteins [43, 44].

Localization of albumin in gut epithelium

Spodoptera frugiperda larvae (5th or 6th instar) were taken from rearing containers and starved overnight. A solid layer of artificial diet was saturated with 100 µl of FITC-albumin at 10 mg/ml. Starved larvae were placed on treated diet and allowed to feed for 5 hours. Larvae that had fed on a large quantity of the diet were selected for immunofluorescence labeling.

The midgut epithelium was left largely undisturbed during extraction for immunofluorescence procedures, to maintain cell morphology. The dissected midguts were placed immediately into formalin-acetic-alcohol (FAA) and stored at 4°C until samples were

embedded in paraffin and sectioned using a microtome. The sections were placed on glass slides and heated to 60°C before being soaked in xylene for 5 minutes, three times to remove paraffin. Following deparaffinization slides were dehydrated in decreasing ethanol baths (100, 75, 50, 25%) for 1 minute each, with a final deionoized water bath.

Sections of FAA fixed midgut tissue were blocked for 1 hour at room temperature in PBS and 5% goat serum. Primary antibody rabbit anti-BSA (Invitrogen) was incubated at a 1:1000 dilution on tissue sections overnight at 4°C. The secondary antibody, goat anti-rabbit Alexa Fluor 647 (Invitrogen) was incubated for 1 hour at room temperature at a dilution of 1:4000. Slides were kept in the dark during staining, so that the sensitive fluorophore would not be depleted. Control immunofluorescence images of tissue from unfed larvae displayed a dramatic increase in auto-fluorescence due to the FAA fixation, which confounds our ability to detect the FITC label. Hence, fluorescent antibodies are required for detection. Tissues from larvae not fed protein were incubated with or without primary antibody to determine non-specific binding.

Statistical analysis

All physiological data were compared by the nonparametric Rank Sum test using Sigmaplot version 12.5 software (Systat Inc, San Jose, CA) and presented as mean ± standard error (SE). A value of $P \leq 0.05$ was considered to be significant. Statistical analysis was performed to test for significant differences in the median values between time points of the same protein and between protein flux rates. Treatment means (i.e. drug inhibitors) were compared to the no treatment samples to test for statistical differences that would indicate an effect on protein movement.

Results

Binding of test proteins to midgut BBMVs

To assess the binding of VP4-P-eGFP and albumin to lepidopteran epithelial proteins, two-dimensional gel electrophoresis was used to separate BBMV proteins isolated from the guts of larvae in 6th instar. Antibodies for albumin, and eGFP did not bind BBMV proteins when ligands were not present. We showed that albumin bound to multiple *Spodoptera* midgut membrane proteins (Fig 2). eGFP used as a control for the structural protein VP4-P-eGFP, showed no binding (Fig 2). Data for VP4-P-eGFP are currently pending.

Protein transport across the midgut epithelium

Previous work demonstrated that both albumin and the whole virus JcDNV cross the gut epithelium of some insect species. Here we investigated the protein flux of albumin and the JcDNV major structural protein, VP4 fused to eGFP. The flux of FITC-albumin and VP4-P-eGFP across the midgut of *S. frugiperda* was investigated in the lumen-to-hemocoel direction, to simulate an *ex vivo* oral delivery pathway.

Albumin: Thirty minutes after introduction into the hemocoel chamber, albumin flux increased linearly with time (Fig 3A). The uneven crossing at early time points may result from the tissue equilibrating to buffer conditions, or retention of albumin in the tissue. After 2 hours albumin was detected in the hemocoel chamber at 525 ± 15 pmol/cm^2. Protein flux after 2 hours was greater than at 10 minutes (*P=0.038* N=6; Rank Sum test). The midguts exposed to FITC-albumin were removed from the chamber system at this time, washed in hemolymph buffer and

examined for albumin retention. While albumin found in the hemocoel and lumen remained intact, the protein was degraded in the tissue (Fig 4).

JcDNV VP4: To investigate the ability of JcDNV VP4 to cross the gut epithelium, the fusion protein VP4-P-eGFP was tested in the Ussing chamber. As *E. coli* expression of this fusion protein was unsuccessful, VP4-P-eGFP was expressed in a baculovirus expression system. Expression of this protein in SF9 cells was easily monitored via eGFP fluorescence, and the recombinant protein was found to cluster in large fluorescent bodies within cells after 24 hours (Supplementary Fig. 2). VP4-P-eGFP was insoluble and a denaturing purification protocol followed to recover the fusion protein. Denaturation and refolding did not appear to negatively affect the fluorescence of the construct, and it was estimated that 80% of VP4-P-eGFP was recovered after refolding.

Uptake of 3 µM eGFP (32.7 kDa) or VP4-P-eGFP (78 kDa) via the gut lumen and subsequent flux across the gut epithelium is shown in Figure 3(B). Only low levels of eGFP were detected in the hemocoel across all time points. The addition of VP4 (47 kDa) fused to eGFP with a proline rich linker significantly enhanced fluorescence in the hemocoel chamber across all time points (*P=<0.017*, eGFP versus VP4-eGFP; Rank Sum Test), with a flux of 118 ± 5 pmol/cm^2 at 2 hours. eGFP crossed the midgut at 22 ± 3 pmol/cm^2 at 2 hours (Fig. 3C). In contrast to albumin, VP4-P-eGFP flux did not increase linearly, but was rapid with the majority of protein crossing within the initial 30 minutes. It appeared that VP4 movement became saturated by this time point. VP4-P-eGFP flux increased between the first 10 minute time point and final time point taken at 2 hours (*P=0.022* N=6; Rank Sum test). The initial flux of VP4-P-

eGFP is a characteristic shared with the wild type virus particle that also displayed rapid uptake early in *ex vivo* transport studies [15]. Transport appeared to increase again after the 1 hour mark, possibly indicating a saturation of receptors by 30 minutes and their reemergence on the apical surface again. This result confirms the ability of VP4 to cross the gut epithelium.

The rate of VP4-P-eGFP into the hemocoel chamber was significantly greater than that of eGFP (*P=0.016* N=6; Rank Sum Test) (Fig. 3C). From Figure 3(C), it can also be seen that of the three proteins tested, albumin had the highest rate of flux. However, greater variation in the rate was seen across replicate experiments for albumin in comparison to VP4-P-eGFP.

Albumin localizes to columnar cells and undergoes clathrin-mediated endocytosis

Using immunofluorescence microscopy techniques we examined the midgut epithelium of 6th instar *S. frugiperda* fed continuously on albumin for 5 hours (Fig 5). There was a distinct lack of albumin present in the mucous-secreting goblet cells. Indeed, the protein was found exclusively in columnar cells with a punctate appearance, which may indicate localization within intracellular vesicles. Albumin was also detected at the brush border membranes of cells. There was little to no binding of the antibodies to proteins in the tissue, and minimal fluorescence in the basal lamina resulting from non-specific binding from the primary antibody.

To characterize albumin's flux which appears to be mediated via the columnar cells, endocytic inhibitors were introduced into the lumen chamber 30 minutes prior to protein addition. This pretreatment allowed the drugs to take effect, and knockdown their respective endocytic processes. The effects of four inhibitors on albumin transport are shown in Figure 6. Chlorpromazine (CPZ) selectively blocks clathrin-dependent entry and in 6th instar *S. frugiperda*

we saw a significant effect on albumin flux to the hemocoel, with an 80% reduction (*P=<0.001* N=8; Rank Sum Test). Tannic acid (TA) alters membrane permeability preventing carrier-mediated fluxes of molecules [45-47], and Methyl-β-Cyclodextran (MBCD) disrupts lipid rafts thereby inhibiting endocytosis by receptor proteins within lipid rafts, through the sequestration of cholesterol [48]. The membrane fixing properties of TA exerted a similar ability to block transport of FITC-albumin as CPZ (*P=0.004* N=5; Rank Sum Test). Methyl-β-Cyclodextran (MBCD) also disrupted albumin transport (*P=0.017*, N=5; Ranks Sum test) (Fig. 6A). However, this effect was not as strong as the impacts of CPZ and TA (*P=<0.019*, compared to MBCD; Rank Sum test). These results indicate that albumin movement across the columnar cells of the gut epithelium in lepidopteran insects is largely active transport relying on clathrin-mediated endocytosis (CME) and to an extent, lipid rafts.

JcDNV VP4-P-eGFP transport inhibited by Tannic Acid

Initial Ussing chamber experiments confirmed VP4's ability to efficiently deliver eGFP across the gut lumen, with a rate of 60 pmol/cm^2/2h (Fig 3C). We further explored the entry process for this protein by using the same inhibitors to obstruct entry routes. Using only VP4 we set out to confirm if this protein would exhibit a pathway of entrance, similar to JcDNV. TA at a 100 µM concentration was able to significantly inhibit the transport of VP4-P-eGFP (*P=0.016* N=4; Rank Sum test) (Figure 6B). Unlike albumin, CPZ had no effect on transport and DYN did not have a significant effect on VP4-P-eGFP flux.

Discussion

In order to exploit toxins active within the hemocoel for suppression of insect pests, appropriate delivery systems must be developed. The use of stable, species-selective carrier proteins provides an attractive and environmentally responsible alternative to current agrochemical applications for pest management.

In this study we used an Ussing chamber to determine the efficacy of a viral protein to transport a macromolecule across the gut of the agricultural pest, *S. frugiperda* [15, 36, 49]. We used BSA, which is known to cross the gut of *Heliothis virescens* [50], as a positive control for examination of the transport of VP4 across the *S. frugiperda* gut epithelium.

Two-dimensional ligand blots were used to screen brush border membrane vesicles from *S. frugiperda* midguts for binding partners. We expected albumin to bind multiple BBMV proteins, whereas VP4 would prove more specific. Here we confirmed albumin's ability to bind numerous surface proteins. Notably, albumin bound a number of proteins between 20 and 75 kDa with an alkaline pI. While this result confirms the binding of albumin to multiple surface proteins, receptors that mediate entry into the epithelial cell have not been identified. It cannot be assumed that all proteins bound by albumin act as receptors that mediate transcytosis. However, the ability to bind strongly to multiple proteins could increase the chances of entry by bringing increased amounts of protein into contact with the membrane surface. No binding of eGFP to the BBMV was detected via far western blotting, indicating that this protein does not mediate VP4-P-eGFP flux across the gut. While eGFP could in theory cross the gut via paracellular routes, this has not been investigated. Ligand blots for detection of VP4-P-eGFP

binding to BBMV proteins have been negative to date, which may reflect the transient nature of JcDNV-receptor interactions [15].

Ussing chamber experiments conducted with gut epithelia derived from larvae of *B. mori* (Lepidoptera) demonstrated that albumin uses CME to cross the midgut [36]. In this study, we show that albumin also uses CME to cross the midgut epithelium of *S. frugiperda*, and that transport occurs via the columnar cells. These results were comparable to [36], despite the use of a different lepidopteran species and chamber system. Albumin retained in the tissue after 2 hours was degraded, although albumin remained intact in both the lumen and hemocoel chambers. This result suggests that while capable of transcytosis, some albumin was degraded for use by the epithelial cells.

The coat protein of a Luteovirus was used to demonstrate the potential use of virus structural proteins to deliver toxins into the hemocoel of agriculturally important aphid pests [14]. To build from this work, we cloned and expressed the coat protein of the lepidopteran parvovirus, *Junonia coenia* densovirus. This virus is unique in its ability to bypass the insect midgut and replicate in underlying tissues. Wang et al [15] demonstrated the ability of JcDNV to rapidly traverse the midgut of *S. frugiperda*. To establish the potential use of the densovirus structural protein in agricultural applications, a single coat protein (VP4) was fused to eGFP. eGFP acted as a fluorescent reporter of protein movement and to determine VP4's ability to transport fused macromolecules. In the absence of VP4, eGFP crossed the gut epithelium into the hemocoel at very low levels. Addition of VP4 fused to the C terminus of eGFP with a polyproline linker significantly enhanced movement at all time points, seen as an increase in

fluorescence in the hemocoel chamber over two hours. Flux facilitated by VP4 was efficient, with the majority of eGFP present in the opposite chamber within the first 30 minutes. VP4-P-eGFP appeared to reach saturation by this time and later began to increase again after 1 hour. This saturation effect could indicate binding to a low abundance membrane protein which needs to be recycled to the membrane for further transport of VP4. In contrast, albumin flux increased linearly over time with no indication of saturation. In contrast to VP4, which we hypothesize binds to a single receptor, albumin may bind multiple surface receptors allowing for exploitation of different transport pathways. The use of multiple transport pathways may explain why saturation was not seen for this protein.

Drugs were used to inhibit the transepithelial flux of VP4-P-eGFP and of eGFP, to delineate entry mechanisms used by the single coat protein, VP4. Albumin entered the epithelial cell by a clathrin-dependent pathway, but was partially degraded within the cell. In contrast, there was no change in VP4-P-eGFP transport following treatment of the epithelium with CPZ, indicating that entry is not clathrin-mediated. CME is considered a constitutive major pathway responsible for the internalization of nutrients and macromolecules, triggered by receptor binding [51]. TA treatment of the apical membrane surface significantly blocked VP4-P-eGFP transport. TA acts by crosslinking surface proteins, preventing endocytosis into vesicles [52]. This result indicates that the protein needs membrane scission to cross the midgut. JcDNV movement across the *S. frugiperda* midgut was also shown to be inhibited by TA [15]. DYN at 100 µM did not impact transport of VP4-P-eGFP. However, Wang et al [15] did not see significant regulation of JcDNV transport until 400-800 µM of DYN was used. A dose dependent response to the amount of inhibitor was also shown for the virus [15], and experiments carried

out with increasing inhibitor concentrations will help determine if VP4 transport is also dynamin-dependent.

Whether VP4 is able to bind to the same receptor as JcDNV is uncertain, although it has been found that VP4 virus-like particles mimic JcDNV entry into LD652 cells [53]. The receptor for JcDNV (or VP4) has not been elucidated. Discovery of the binding partner for this virus could further our ability to exploit the nature of this virus for pest control. Here we have demonstrated that VP4-P-eGPF, which does not produce VLPs, can cross the midgut via clathrin-independent endocytosis. Figure 7 shows the proposed entry mechanisms of albumin, and VP4-P-eGFP into the gut epithelial cells of S. frugiperda based on the results of the current study. Having established that VP4 can carry a protein across the midgut, further work will focus on *in vivo* VP4 mediated delivery of a toxin, and assessment of resulting *S. frugiperda* mortality.

Based on our results, JcDNV VP4 shows promise for delivery of insect specific toxins into the hemocoel of *S. frugiperda*. Given the resistance to Bt toxins reported in this species, novel approaches for management are of particular importance.

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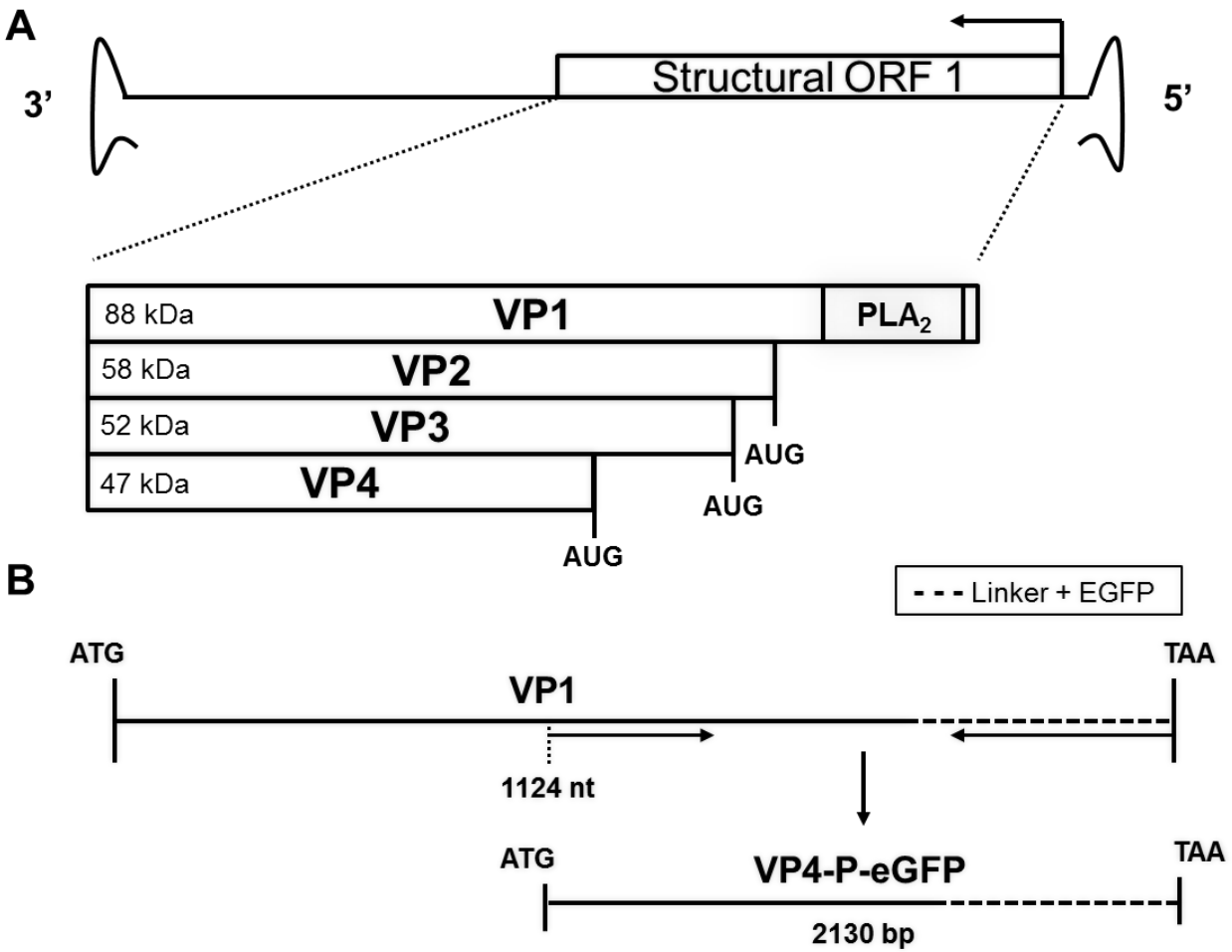


Figure 1. Production of JcDNV structural proteins. **(A)** An open reading frame encodes the structural protein VP1, which contains a Phospholipase A_2 (PLA_2) region. VP2 to VP4 are produced by leaky scanning, and have the same C terminal region. **(B)** VP4-P-eGFP was amplified from VP1-P-eGFP using PCR resulting in a 78 kDa protein. The positions of forward and reverse primers (VP4 F and VP4 R) are indicated by black arrows.

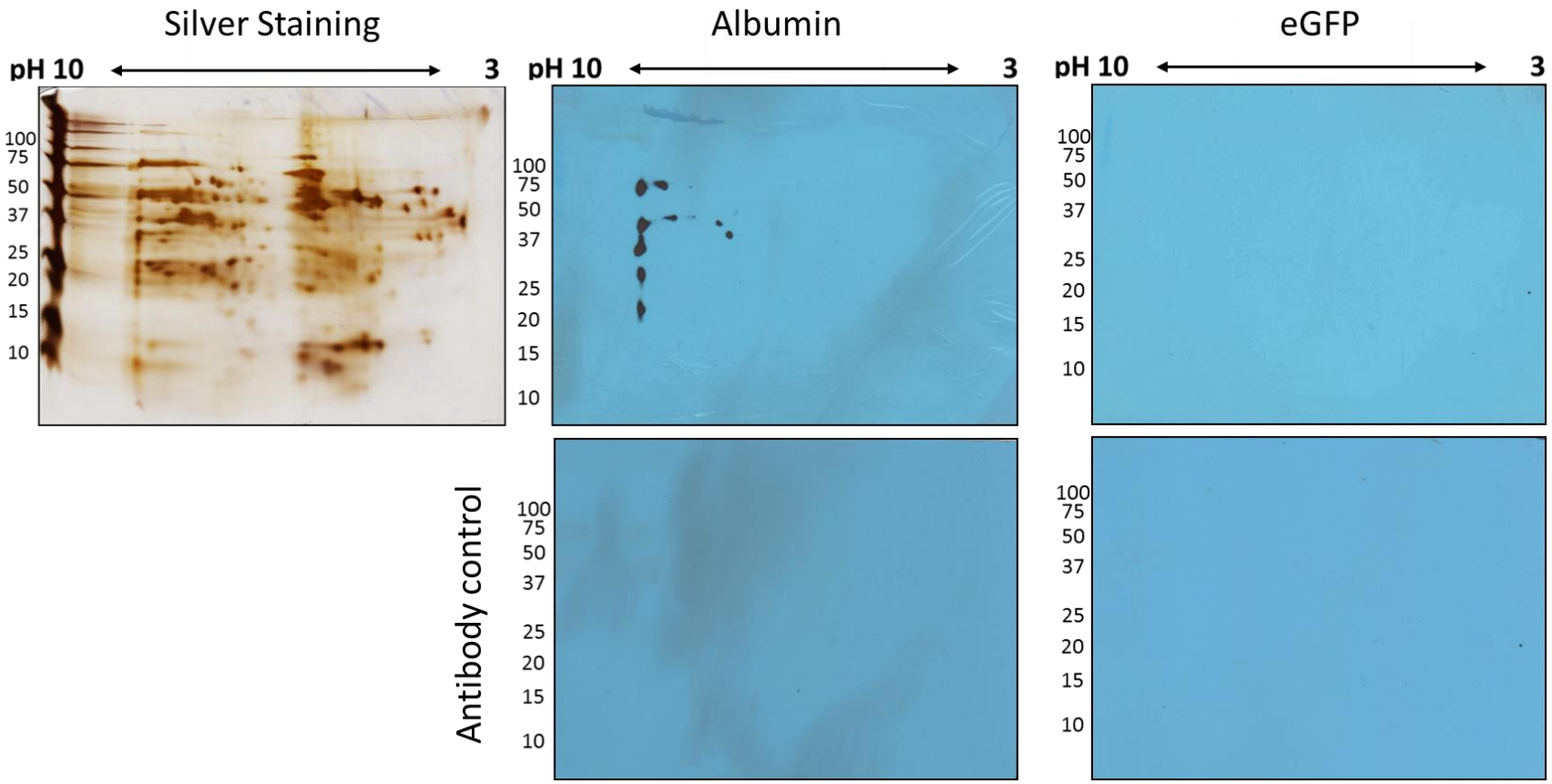


Figure 2. Binding of albumin and eGFP to *S. frugiperda* BBMV. Far western blotting (pH 3-10) was conducted in the presence or absence of albumin and eGFP. Antibody only controls were used to detect non-specific antibody binding. A silver stained two-dimensional SDS-PAGE gel depicts 50 µg of BBMV. Molecular mass markers are indicated on the left of each blot. VP4-P-eGFP results are pending.

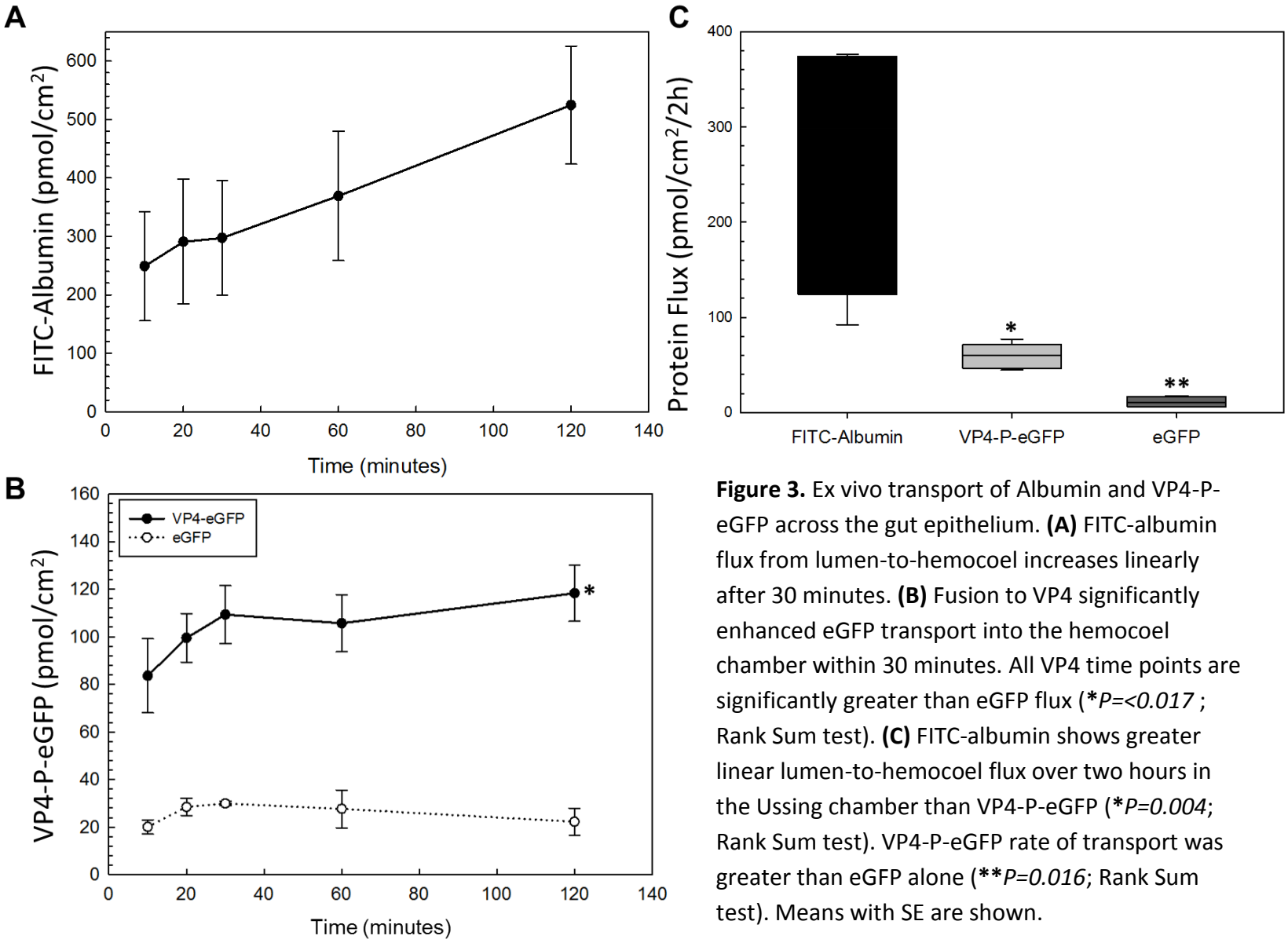


Figure 3. Ex vivo transport of Albumin and VP4-P-eGFP across the gut epithelium. **(A)** FITC-albumin flux from lumen-to-hemocoel increases linearly after 30 minutes. **(B)** Fusion to VP4 significantly enhanced eGFP transport into the hemocoel chamber within 30 minutes. All VP4 time points are significantly greater than eGFP flux (**P=<0.017* ; Rank Sum test). **(C)** FITC-albumin shows greater linear lumen-to-hemocoel flux over two hours in the Ussing chamber than VP4-P-eGFP (**P=0.004*; Rank Sum test). VP4-P-eGFP rate of transport was greater than eGFP alone (***P=0.016*; Rank Sum test). Means with SE are shown.

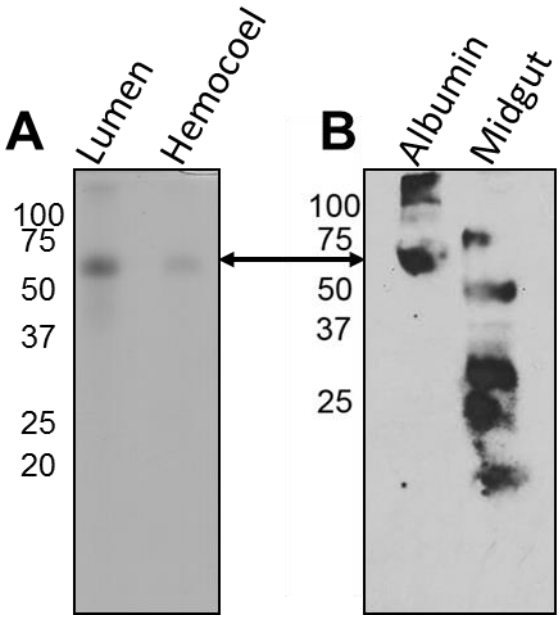


Figure 4. The integrity of albumin in the Ussing chamber and in midgut tissue. **(A)** After 2 hours in the respective chambers, albumin remained intact with no degradation detected in a 12% SDS-PAGE gel. The gel was stained with Coomassie Blue. **(B)** Albumin retained in the midguts after 2 hours, was degraded as detected by western blot (anti-BSA antiserum). Arrow indicates expected size of albumin (66 kDa).

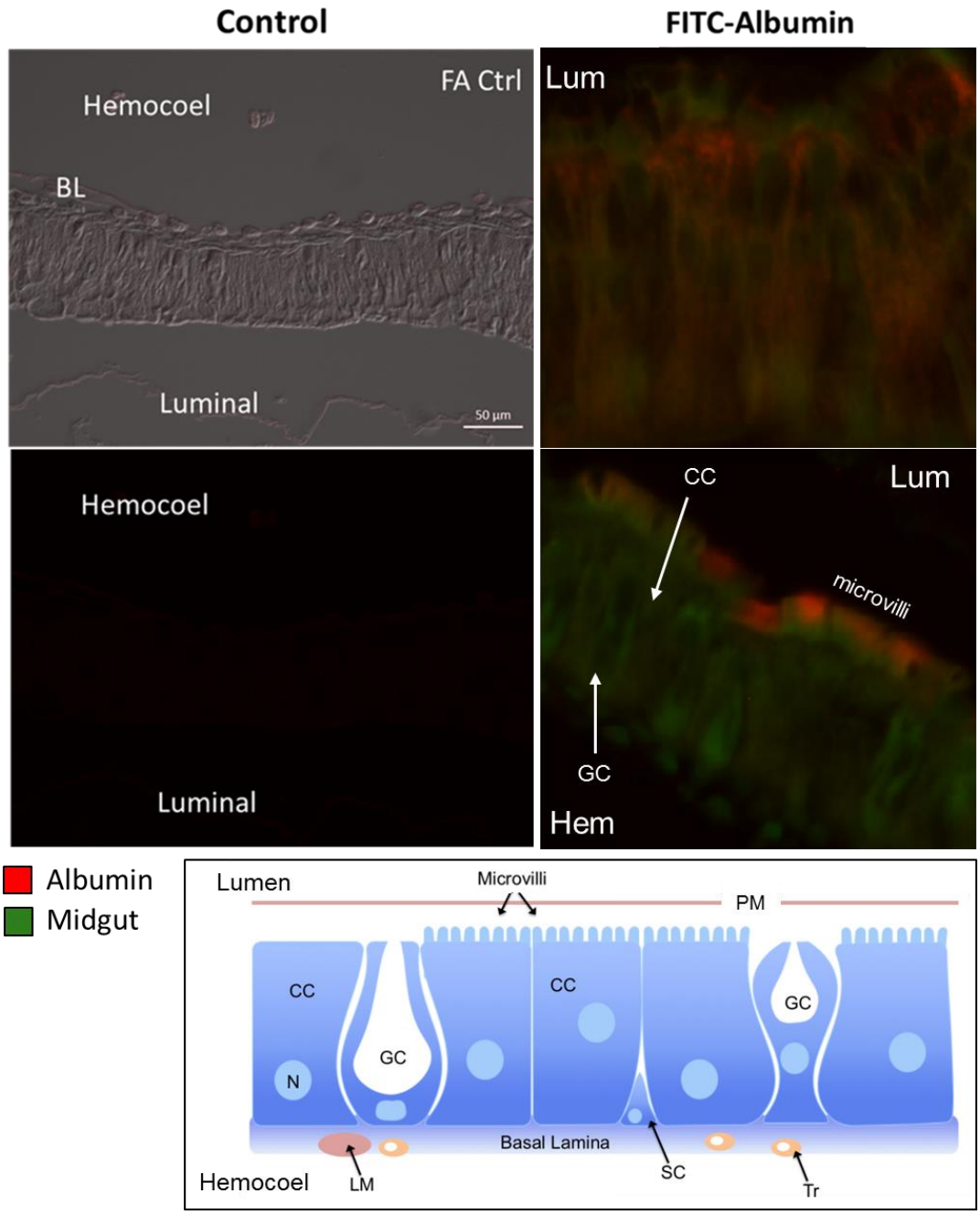


Figure 5. Immunofluorescence detection of albumin following uptake into the *S. frugiperda* gut epithelium. Sections from larvae fed on diet only (control: light and fluorescence images) and FITC-albumin are shown. Albumin (red fluorescence) was detected within microvilli and within the tissue, but not in goblet cells. Due to FAA fixation, midguts were highly auto-fluorescent, shown here as green. A schematic of the insect midgut cell morphology is provided. FA Ctrl (FITC-albumin control), Lum (Luminal), Hem (Hemocoel), CC (Columnar cell), GC (Goblet cell), BL (Basal lamina), SC (Stem cell), Tr (Trachea), PM (Peritrophic membrane), LM (Lateral muscle), N (Nucleus)

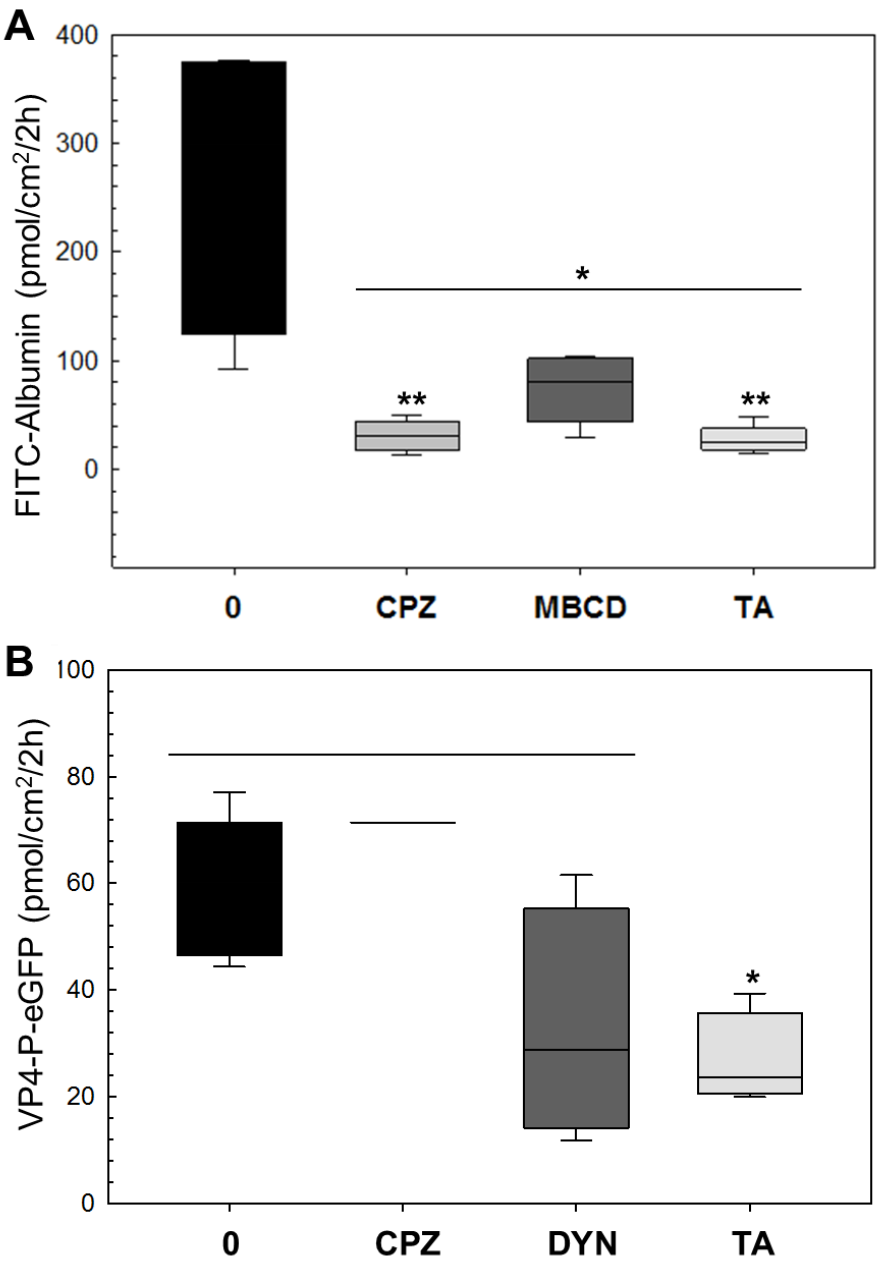


Figure 6. Inhibitors block FITC-albumin and VP4-P-eGFP flux. **(A)** Albumin undergoes clathrin-mediated endocytosis. CPZ and TA inhibited albumin transport across the midgut indicating that albumin uptake is mediated by clathrin-dependent endocytosis. Mean ± s.e. is significantly different from no inhibitor (**P=<0.017)* or MBCD (***P=<0.019*); Rank Sum Test. **(B)** Inhibitor of membrane fusion blocks VP4-P-eGFP endocytosis. TA significantly knocked down VP4-P-eGFP transport, and no effect was seen with CPZ. Based on published data [53], higher concentrations of DYN need to be tested. Mean ± s.e. is significantly different from no inhibitor (**P=0.016*); Rank Sum test. Inhibitor concentrations: CPZ (100 µM), TA (100 µM), DYN (100 µM), MBCD (10 mM).

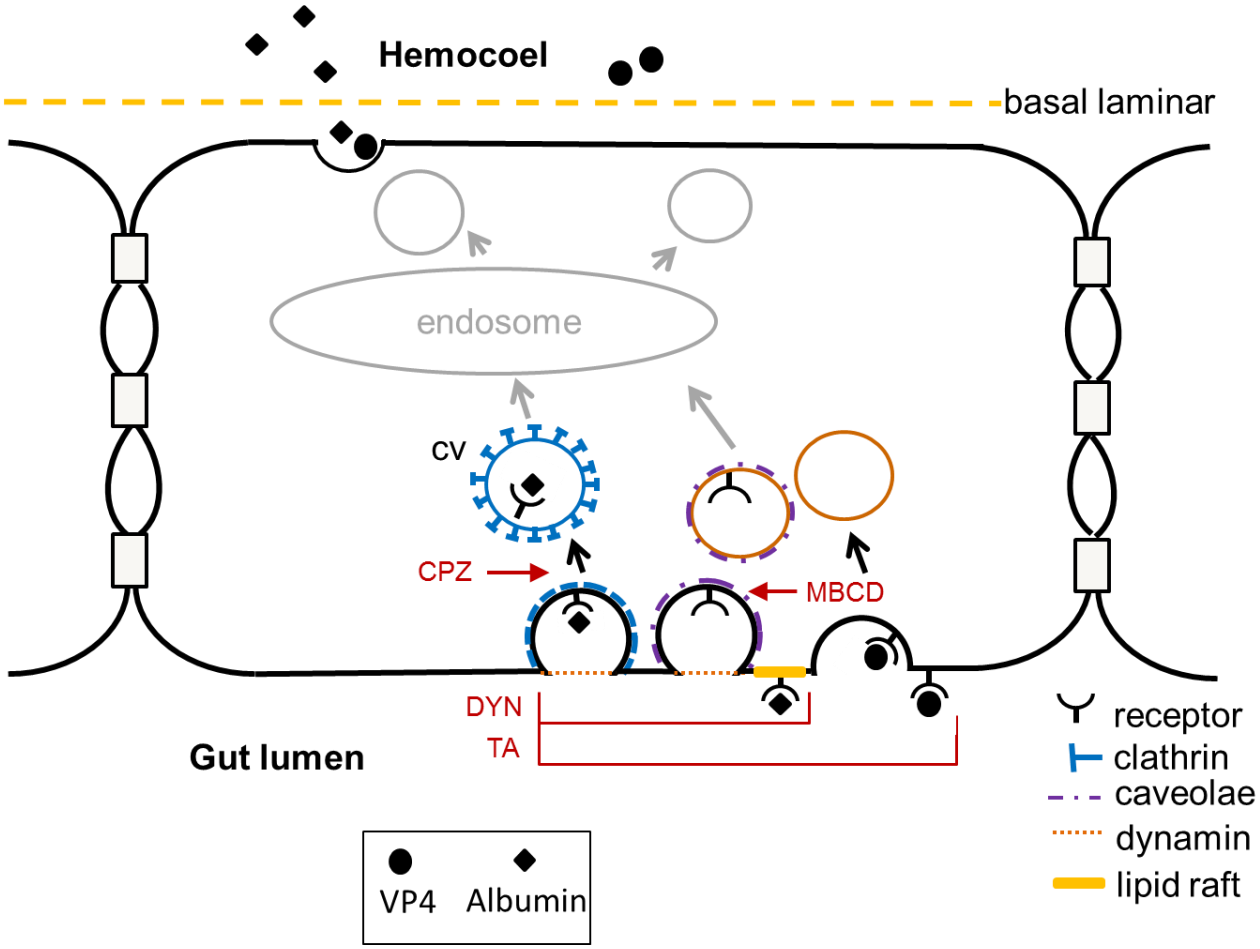
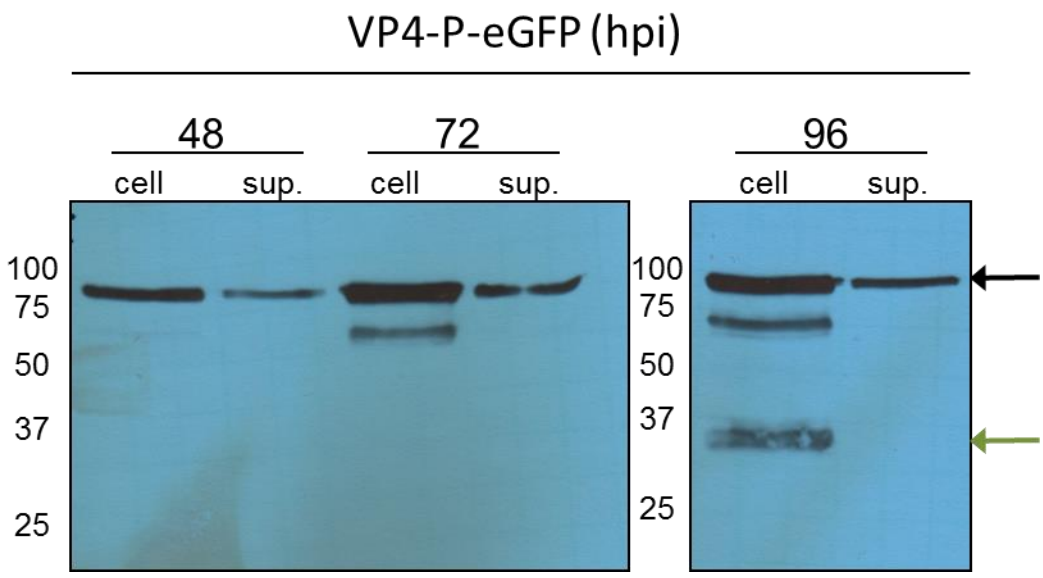
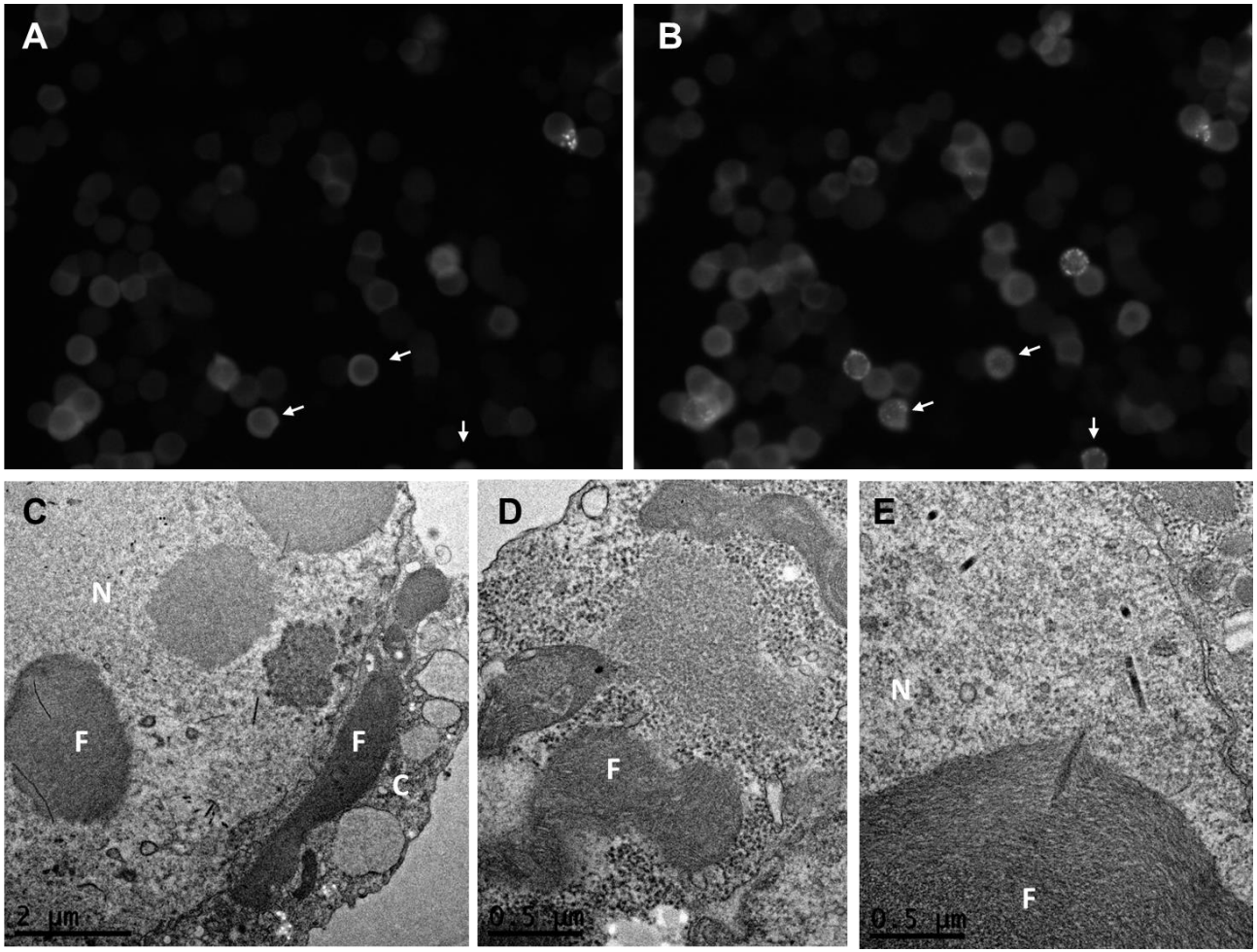


Figure 7. Schematic of expected entry mechanisms of Albumin and VP4-P-eGFP, including inhibitor actions. Both albumin and VP4-P-eGFP transport from the lumen into the hemocoel. TA (inhibits all endocytic mechanisms) prevented the entry of Albumin and VP4-P-eGFP, indicating that cellular endocytosis into vesicles is required to cross into the hemocoel. Only albumin was affected by CPZ and MBCD (inhibits CME and disruption of lipid rafts, respectively). It is expected that VP4 binds an unknown receptor and uses clathrin-independent entry. Albumin may bind multiple receptors that are mainly taken up via CME.



Supplementary Figure 1. Baculovirus expression of VP4-P-eGFP in SF9 cells. VP4-P-eGFP primarily localized to SF9 cells, with no degradation observed at 48 hpi. Degradation was observed at later times post infection. The 72 hpi time point was selected as the optimal harvest time. At 96 hpi a lower band expected to be eGFP was seen. Black arrow denotes expected size of VP4-P-eGFP. Green arrow indicates degraded band at expected size of eGFP (32.7 kDa). Cell – protein from lysed SF9 cells; Sup. – supernatant analyzed after centrifugation of cells. (Anti-eGFP 1:5000)



Supplementary Figure 2. Baculovirus expression of JcDNV VP4-P-eGFP in SF9 cells. **(A)** Light microscopy of SF9 cells expressing VP4-P-eGFP 12 hours post infection (hpi) and **(B)** the same cells 24 hpi. Punctate fluorescence was seen indicative of insoluble VP4-P-eGFP. Some cells exhibit fluorescence without clustering, these are in the early stages of protein production. Arrows denote cells that developed punctate structures over 12 hours. **(C, D, E)** Transmission Electron Microscopy of baculovirus-infected SF9 cells expressing VP4-P-eGFP 72 hpi. Fibrillar bodies (F) are shown in both nuclei (N) and cytoplasm (C). While baculovirus produced fibrillar bodies are expected in the nucleus, they are not expected in the cytoplasm. As expected, no VLPs were observed in cells infected with the baculovirus expressing VP4-P-eGFP.

CHAPTER 3: CONCLUSIONS

Insecticidal neurotoxins can be found from a number of different sources (microbes, spiders, parasitoid wasps and entomopathogenic nematodes) and expression of hormones and peptides derived from insects can interfere with important biological functions. Importantly, these bioinsecticides have a range of unique modes of action specific to insect target sites, making them effective for pest control and safe for human consumption. However, many of these agents have not been employed due to their inability to reach the hemocoel when delivered orally. The insect gut poses the largest obstacle to overcome, and appropriate delivery systems are needed to take advantage of these novel biocontrol agents.

Albumin

Albumin is a serum protein reported to cross the gut of *Heliothis virescens* and *Bombyx mori* via clathrin-mediated endocytosis [1]. Here we have found that albumin also crosses the *S. frugiperda* midgut in a similar manner. We used albumin as a positive control to first, establish our Ussing chamber system as a viable option for studying protein movement in the insect gut, and second to compare with JcDNV structural protein VP4. To this end, we have shown that albumin undergoes receptor-mediated endocytosis and transcytosis, potentially using multiple receptors. Addition of inhibitors revealed that albumin relied on clathrin-dependent entry, but the use of Methyl-β –Cyclodextran (MBCD) also indicated possible lipid raft-mediated entry. MBCD prevents lipid raft protein endocytosis by removing cholesterol from the membrane, but can lack specificity [2]. Whether this had an effect on CME and other pathways is unclear, but is

a possibility. Albumin would not be appropriate for toxin delivery as it crosses many epithelial layers in humans [3]. Albumin and artificial mutants have potential applications in human medicine, and have been used to deliver and extend the half-life of clinical drugs [3]. In the agricultural setting albumin could be used to enhance delivery of viruses and toxins; however restriction to invertebrates would be questionable.

Virus Structural Proteins as Toxin Delivery Agents

Viruses have evolved to exploit cellular pathways to enter cells, or cross tissue layers to establish infections in underlying tissues. Notably, some plant viruses traffic across the gut of aphids and other sap sucking insects to reach the salivary glands for transmission. *Junonia coenia* densovirus (JcDNV) selectively infects lepidopteran species [4] and can cross the midgut without undergoing replication [5]. The limited host range of JcDNV and efficiency of VP4 transport across the midgut are advantageous for the use of VP4 to deliver neurotoxins to key agricultural pests, avoiding impacts on non-target species. There are also a number of proteins able to transcytose epithelial barriers to reach underlying organs or tissues [3, 6-8].

In Chapter 2, we set out to determine the capability of a JcDNV structural protein to cross the midgut of the economically important pest, *Spodoptera frugiperda*. The JcDNV capsid is comprised of 60 proteins, of which VP4 makes up the majority of polypeptides. *Ex vivo* Ussing chamber results indicated that VP4 greatly enhanced the flux of the large molecule, eGFP (32.7 kDa) across the *S. frugiperda* gut epithelium. This was significant across all time points recorded.

Sivakumar et al [9] expressed Pea enation mosaic virus coat proteins in SF9 cells and found fully formed VLPs using transmission electron microscopy (TEM). We also used TEM to look for VLP formation in SF9 cells infected with a baculovirus expressing VP4-P-eGFP at 72 hours post infection (hpi) that were positive for eGFP fluorescence. VLPs were not detectable in any of the cells, but large fibrous bodies were seen in both the cytoplasm and the nucleus. Those in the nucleus are consistent with baculovirus infections. The fibrous structures in the cytoplasm may correlate with the fluorescent clusters that were detected 24 hpi. VP4 is capable of particle assembly when expressed in insect cells [10, 11]. The lack of VLP detection likely results from attachment of eGFP to the C terminus. All four coat proteins share the same C terminal region which may be important in capsid formation. Addition of the linker and protein impair particle formation by VP4, as seen for the structural proteins of other viruses [9].

JcDNV uptake mechanisms have been investigated in both *in vivo* and *ex vivo* systems [11, 12]. In LD652 cells, JcDNV was dependent on clathrin-mediated endocytosis (CME) [11]. However, in *S. frugiperda* midgut cells transport was distinctly clathrin-independent, relying on dynamin mechanisms [12]. Adeno-associated virus (AAV) is a vertebrate parvovirus that uses distinct receptor(s) for infection and another unknown receptor(s) for transcytosis [13, 14]. Similarly, HIV exploits multiple receptors for different purposes [15]. AAV and porcine parvovirus use more than one route and take advantage of more than one cellular mechanism [13, 16]. JcDNV, an arthropod parvovirus, could act similarly and utilize different receptors and mechanisms to undergo transcytosis or infection. Clathrin vesicle entry of JcDNV and VP4-VLP into LD652 gypsy moth cells lead to late endosomal trafficking and infection [11]. This is distinct

from what seems to occur in midgut cells, where JcDNV was trafficked to the basolateral surface in a dynamin-dependent manner without replication [12]. Delineation of internalization mechanisms in other *S. frugiperda* tissues where JcDNV can establish an infection should be done. Perhaps JcDNV will use CME to enter the trachea and epidermal cells. Despite research into entry mechanisms and pathogenicity the receptor for JcDNV is currently unknown.

JcDNV and *Galleria mellonella* densovirus (GmDNV) share 96% sequence homology, but have distinct host ranges. JcDNV is actually incapable of crossing the midgut of *G. mellonella*, the sole host of GmDNV [4]. Comparison of capsid topology revealed that these viruses only retain minor differences with great surface similarities [17]. Bruemmer et al [17] identified 8 amino acids altered between GmDNV and JcDNV. These 8 residues are located in the five-fold axis, around the three-fold axis peaks, and the dimple of the two-fold axis [17, 18]. When substituted for GmDNV residues, it was found that the mutations did not have a detrimental effect on cell entry or success of infection in underlying tissues [18]. However, the mutated capsids did affect midgut tropism, and appeared to be governed by the five-fold axis. This indicated that mechanisms used to transcytose barriers are different from those used for infection. The surface of JcDNV is highly disordered, but cryo-electron microscopy reconstructions found that the five-fold axis canyon harbors 21 N-terminal residues of VP4 [17]. VP4 comprises 41 of the 60 polypeptides that make up the capsid, and in Chapter 2 we showed that it is capable of crossing the midgut without VP1-3. Together with its location in the five-fold axis it would therefore not be unreasonable to suggest VP4 as a part of the receptor recognition site for JcDNV. It should be noted that VP4 when expressed alone, may not be able

to bind the same receptor as the intact JcDNV virion. JcDNV could rely on interactions between multiple capsid proteins or specific structures to bind, which may be absent from VP4. Additional ligand blots with JcDNV could be done in parallel with VP4 to determine if the virus and protein bind similar BBMV proteins.

Future Research

The current understanding of protein movement across the insect gut is limited compared to mammalian systems. In Chapter 2, we demonstrated that a viral coat protein could sufficiently cross a lepidopteran barrier, and crossing one insect system is not indicative of a protein's behavior in another insect. Greater understanding of mechanisms and processes exploited by viruses and proteins would facilitate their utility as vectors to control insects that damage crops and spread plant and human pathogens. Potential avenues for future research include:

1. *Create VP4-toxin fusion and perform bioassays with S. frugiperda larvae to test for mortality.* Now that VP4 has been shown to cross the midgut, the next step is to fuse the coat protein to a toxin, such as Hv1a and determine its efficacy *in vivo. S. frugiperda* are highly susceptible to JcDNV, and it is expected that the coat protein will be stable upon oral delivery.
2. *Identification of VP4 receptor(s).* First, using VP4 and two-dimensional ligand blots or pull-down assays with lepidopteran BBMV, followed by MS/MS protein sequencing to identify bound proteins. Following would be the expression of putative receptors in insect cell cultures for detection of *in vitro* binding of VP4 to the putative receptor.

JcDNV virions would also be used to investigate if the virus and the protein exhibit similar binding patterns.

3. *Test for encaspidation and delivery of dsRNA via VP4 VLP to lepidopterans.* Ussing chamber experiments with only JcDNV VP4 show a similar pattern of transport to the wild-type virus, with rapid uptake in the first 30 minutes. This result is indicative of the virus' need to cross efficiently when it encounters the gut to avoid removal from the insect by cell sloughing. When the four coat proteins of JcDNV are expressed individually, VP4 can form virus-like particles (VLP) without the addition of other proteins [10, 11]. VP4's ability to self-assemble could in theory encapsidate double-stranded RNA for RNAi-mediated control of lepidopteran. The *Aedes aegypti* densovirus (AeDNV) was used as a vector to deliver single-hairpin RNA and could successfully induce RNAi-mediated mortality in mosquito larvae [19]. Lepidopterans are notoriously difficult targets for RNAi control as dsRNA is unable to penetrate the cytoplasm [20]; vectors that can infiltrate cellular barriers, protect RNA from degradative enzymes and enter the cytoplasm are greatly needed.
4. *Test transport of teratocyte secretory protein 14, and other proteins of interest in the Ussing chamber.* TSP14 is a small (14 kDa) Cys-motif protein secreted from the teratocytes of a parasitoid wasp (*Microplitis croseipes*), and inhibits the growth and development of lepidopteran larvae [21, 22]. Polydnaviruses are viral endosymbionts of parasitoid wasps and also encode Cys-motif genes that are host translation-inhibitory factors (HTIF) [23]. These wasp and viral proteins are potent insecticides when injected, but importantly, also have efficacy when fed to insect larvae [22, 23]. The efficiency of

transport of HTIF, TSP14 and other proteins could be assessed in the Ussing chamber, as we have done with VP4 and albumin.

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